Plant Metabolic Engineering

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Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, improve the production of existing compounds, or mediate the degradation of compounds. In highlighting progress in plant metabolic engineering over the past 25 years, it is first important to stress that it is in fact quite a young science in plants. Our knowledge of substrate-product relationships in plant pathways was reasonably well advanced by 1975 as a result of the application of radiolabel tracer studies during the previous decades. Attempts to use this knowledge to engineer metabolism in plants, however, first required the development of basic molecular biological technologies such as cloning, promoter analysis, protein targeting, plant transformation, biochemical genetics, and other areas of plant biology (described elsewhere in this volume). Despite this delay significant progress has been made since the mid-1980s in the molecular dissection of many plant pathways and the use of cloned genes to engineer plant metabolism. Although there are numerous success stories, there has been an even greater number of studies that have yielded completely unanticipated results. Such data underscore the fragmented state of our understanding of plant metabolism and highlight the growing gap between our ability to clone, study and manipulate individual genes and proteins and our understanding of how they are integrated into and impact the complex metabolic networks in plants. With an estimated 100,000 unique compounds produced in the plant kingdom, elucidating these metabolic networks is likely to be an exciting endeavor. The few examples cited in this article are meant to highlight common themes that have emerged in the field of plant metabolic engineering, to exemplify the advancements and limitations of current approaches and to provide a forward looking perspective of this exciting area of plant biology over the coming years.

TECHNOLOGICAL ADVANCEMENTS IN GENE DISCOVERY HAVE HELPED TO DRIVE METABOLIC ENGINEERING

The dependence of progress in plant metabolic engineering upon technological advancements is beautifully exemplified by research in lipid metabolism. Though much was learned at the biochemical level about individual steps of plant lipid synthesis during the 1970s and early 1980s, progress in purifying and cloning many pathway enzymes, especially those that are membrane associated, was hindered due to biochemical difficulties inherent in the target enzymes. A major breakthrough in the field came from genetic dissection of the pathway in Arabidopsis (2). This pioneering work in plant biochemical genetics was modeled after mutation-based approaches to study metabolism in bacterial systems. Earlier work by Somerville and coworkers studying photorespiration in Arabidopsis proved the feasibility of using biochemical genetics to dissect plant pathways (24). For lipid biosynthesis, over 10,000 mutated Arabidopsis plants were screened by gas chromatography for altered fatty acid profiles. This resulted in identification of a suite of novel and informative mutations defining steps of the lipid biosynthetic pathway, which allowed genetic models of the plastidic and extraplastidic pathways to be developed and tested (2). The same mutants provided genetic targets for subsequent cloning of several pathway genes by chromosome walking, T-DNA tagging, and various homology-based screening approaches (18). Our understanding of the biosynthesis of other classes of plant compounds such as amino acids, waxes, anthocyanins, and ascorbic acid has been similarly advanced by analogous molecular genetic approaches dissecting their respective pathways (5, 6, 13, 19, 20).

A second example of technology's impact on gene discovery for plant metabolic engineering comes from work on the carotenoid biosynthetic pathway. Though the pathway in plants had been known since the mid 1960s, the labile, membrane-associated enzymes remained recalcitrant to isolation and study. However, because carotenoids are also synthesized by many photosynthetic and non-photosynthetic bacteria, the development of molecular genetic tools in prokaryotes during the 1980s allowed plant researchers to access carotenoid biosynthetic genes from prokaryotes. Integrating prokaryotic systems into their work enabled researchers to finally clone the majority of carotenoid biosynthetic enzymes from plant during the 1990s (for review, see 7).

One general approach used mutant complementation to identify and isolate carotenoid biosynthetic genes based on their resistance to specific herbicides. An early success was the cloning of phytoene desatu-

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rase (PDS) from the cyanobacterium *Synechococcus-PCC7942* (4). Mutants resistant to an herbicide that inhibits PDS activity were first selected, and a library of the mutant DNA were transformed into wild-type *Synechococcus.* The mutant PDS gene was identified by its ability to confer herbicide resistance in the wild-type background. The evolutionary relationship of cyanobacteria and plants quickly allowed isolation of PDS orthologs from a number of plant species. A second approach, termed color complementation, engineered carotenoid biosynthetic genes from bacterial, fungal, and plant sources onto a single plasmid for expression in *Escherichia coli*, which normally lacks endogenous carotenoids and the associated enzymes. Depending on which genes and portion of the pathway were engineered, accumulation of variously colored pathway intermediates resulted. Transformation of plant cDNA expression libraries into such *E. coli* backgrounds allowed functional identification of the rare cDNAs (often one in several hundred thousand) encoding the next enzyme of the pathway based on the associated change in color of the carotenoid product. Similar strategies utilizing heterologous systems have also allowed function-based cloning of enzymes for the synthesis of plant sterols, amino acids, and vitamins (11, 20, 22, 23).

PREDICTING THE OUTCOME OF METABOLIC ENGINEERING IS A CHALLENGING JOB

Although progress in pathway gene discovery and our ability to manipulate gene expression in transgenic plants has been most impressive during the past two decades, attempts to use these tools to engineer plant metabolism has met with more limited success. Though there are notable exceptions, most attempts at metabolic engineering have focused on modifying (positively or negatively) the expression of single genes affecting pathways. In general, the ability to predict experimental outcomes has been much better when one is targeting conversion or modification of an existing compound to another rather than attempting to increase flux through a pathway. Modifications to metabolic storage products or secondary metabolic pathways, which often have relatively flexible roles in plant biology, have also been generally more successful than manipulations of primary and intermediary metabolism (16, 26). Some brief examples follow.

As was the case for gene discovery, the lipid biosynthetic pathway was one of the earlier pathways to be targeted for manipulation and represents one of the better examples of metabolic engineering in plants to date. Most enzymes for fatty acid synthesis in plants have been cloned and various academic and industrial groups have modified their expression to manipulate oilseed fatty acid composition. Space permits only a single example to be discussed here and the reader is referred to the Somerville article for additional discussions. The engineering of soybean and canola to produce higher levels of mono-unsaturated fatty acids was undertaken because their oils contain high levels of linolenic acid (18:2), which is susceptible to oxidation and limits the shelf life and utility of these oils. Antisense inhibition of oleate (18:1) desaturase expression resulted in oil that contained >80% oleic acid (a mono-unsaturated fatty acid) and had a significant decrease in polyunsaturated fatty acids (12). The resulting mono-unsaturate-rich oils are more stable to oxidation, healthier in the human diet than the corresponding poly-unsaturate containing oils, and represent an excellent early example of the practical application of metabolic engineering in plants.

Other areas of plant metabolism with high potential to benefit human health have also been successfully engineered in recent years (8). In one example, the last enzyme in the synthesis of α-tocopherol, γ-tocopherol methyltransferase (γ-TMT) was used to increase the vitamin E activity of Arabidopsis seed oil (22). Arabidopsis seed, like most oilseed crops, contains a high proportion of γ-tocopherol, which has 10% of the vitamin E activity of α-tocopherol. Expression of γ-TMT in Arabidopsis seed resulted in the conversion of the large pool of γ-tocopherol to α-tocopherol with a corresponding 10-fold increase in vitamin E activity. Engineering similar conversions in soybean, canola and maize would elevate the levels of this important antioxidant/vitamin in the diet and potentially have significant health consequences for the general population (10). In an example of metabolic engineering of plant vitamin content targeted at the developing world, β-carotene (provitamin A) was recently engineered into rice endosperm (29). Vitamin A deficiency is a serious health issue in many parts of the developing world. Rice is a major staple in developing countries, but is a poor source of many essential vitamins and minerals, including β-carotene (provitamin A). Efforts to engineer β-carotene production in rice benefited directly from the identification of carotenoid biosynthetic genes in model systems described earlier. Three carotenoid biosynthetic enzymes (two from plants and one from bacteria) were engineered for simultaneous expression in rice endosperm. The resulting first generation transgenic rice produced yellow endosperm (so-called "golden rice") containing β-carotene at levels that would provide 10% of the recommended daily allowance with an average daily rice intake. Subsequent manipulation may allow the vitamin A recommended daily allowance to be approached with a daily rice intake and potentially provide relief from vitamin A deficiency for millions worldwide.

When faced with the novel experimental possibilities that molecular, genomic, and transgenic approaches have presented over the past two decades, researchers can be tempted to become fixated on

producing transgenic plants and lose appreciation for the important roles enzyme kinetics play at individual reaction steps and within entire pathways. The results of careful consideration of enzyme kinetics in metabolic engineering were elegantly demonstrated in research directed at modifying starch synthesis by manipulating ADP-Glc pyrophosphorylase (ADPGPP). Plant ADPGPP is sensitive to allosteric effectors and has been proposed to be a key regulator limiting starch synthesis. *Escherichia coli* ADPGPP is involved in glycogen synthesis and is also sensitive to allosteric effectors. Mutations affecting allosteric regulation cause an increase in glycogen levels in *E. coli*. Stark et al. (25) engineered wild-type and mutant *E. coli* ADPGPP for expression in plants and assayed the effect on starch accumulation. Tubers from potato plants transformed with the wild-type *E. coli* enzyme had starch levels similar to wild-type plants, whereas those transformed with the allosterically insensitive *E. coli* ADPGPP enzyme had starch levels up to 60% higher than wild type. The effect was only observed when the mutant protein was targeted to the chloroplast and driven by a tuber specific promoter; constitutive expression was lethal. Such results demonstrate the importance of considering the target tissues, subcellular localization, and kinetics of enzymes when engineering plant metabolism.

Attempts to manipulate the Lys content of seeds (Lys is a limiting amino acid in most seeds used for food or feed) illustrate that one needs to consider catabolic, as well as anabolic, variables when trying to engineer a particular metabolic phenotype in plants. A key step in Lys synthesis is carried out by dihydrodipicolinate synthase, which is feedback inhibited by the pathway end product, Lys, and thus plays a key role in regulating flux through the pathway. Engineering plants to overexpress a feedback insensitive bacterial dihydrodipicolinate synthase, similar to the approach with ADPGPP described earlier, greatly increased flux through the Lys biosynthetic pathway. However, in most cases this did not result in increased steady-state Lys levels as the plants also responded by increasing flux through the Lys catabolic pathway (1, 9). Substantial increases in Lys only occurred in plants where flux increased to such a level that the first enzyme of the catabolic pathway became saturated.

The manipulation of well-characterized "rate-limiting" enzymes of primary carbon metabolism to study their role in regulating pathway flux has provided some of the more surprising results from metabolic engineering in plants (for review, see 26). These experiments drive home the point that a thorough understanding of the individual kinetic properties of enzymes may not be informative as to their role in complex metabolic pathways. Potential regulatory enzymes are generally identified based on their catalyzing irreversible reactions and being regulated by appropriate effector molecules for a pathway; traditional biochemical hallmarks of rate controlling enzymes. When the highly regulated Calvin cycle enzymes Fru-1, 6-bisphosphatase and phosphoribulokinase were reduced 3- and 10-fold in activity, respectively, surprisingly minor effects were observed on the photosynthetic rate. In contrast, a minor degree of inhibition of plastid aldolase, which catalyzes a reversible reaction and is not subject to allosteric regulation, led to significant decreases in photosynthetic rate and carbon partitioning. Thus aldolase, an enzyme seemingly irrelevant in regulating pathway flux, was shown to have a major control over the pathway. Analogous surprises were also found when manipulating presumed "rate limiting" enzymes of glycolysis. Such data has called into question many of the longstanding ideas about flux regulation in plants and is forcing a reassessment of the role of individual enzymes in the process. These studies also make clear the caution that must be exercised when extrapolating individual enzyme kinetics to the control of pathway flux.

TRANSCRIPTIONAL REGULATORS MAY ALLOW MANIPULATION OF ENTIRE PATHWAYS

Thus far we have only discussed manipulating structural genes for pathway enzymes to affect changes in metabolism. An intriguing approach for metabolic engineering and increasing our understanding of the coordinate changes in gene expression needed to regulate entire pathways is to identify and study transcriptional factors controlling pathways or branches of metabolism (14, 15, 17, 28). Many of the transcriptional regulators affecting plant biochemistry and development were originally identified by chemical- or transposon-based mutant screens in maize, snapdragon or Arabidopsis. The cloning of such loci has provided the opportunity to use these genes to manipulate plant biochemistry in the host organism or in other plants. One of the early instances of using this approach to manipulate plant biochemistry was the engineering of Arabidopsis to express the maize transcription factors C1 and R, which regulate production of anthocyanins in maize aleurone layers (17). Expression of C1 and R together from a strong promoter caused massive accumulation of anthocyanins in Arabidopsis, presumably by activating the entire pathway. More recently, the maize transcriptional regulators C1, R, and P were expressed in cell cultures and the effect on anthocyanin biochemistry and global gene expression analyzed (3). Novel insights into the anthocyanin pathway, its regulation, and additional differentially expressed targets of these regulatory genes were obtained. Such expression experiments hold great promise and may eventually allow the determination of transcriptional regulatory networks for biochemical pathways.

ELEPHANTS, BLIND MEN, AND A BRIEF DIP IN THE METABOLIC POOL

This article is too brief to effectively cover many aspects of metabolism and metabolic engineering in plants during the past 25 years. Suffice it to say, it has been a tremendously exciting quarter century that has set the stage for a revolution in the way we think about and approach metabolism and metabolic engineering in plants. The pace of gene discovery in plant metabolism has increased dramatically during the past decade and will only quicken in coming years as the public deposition of expressed sequence tags and complete genomes allows plant researchers to move their pathways and experiments with increasing ease between organisms and through evolutionary time. During the last 15 years we have also refined our ability to engineer changes in gene expression in transgenic plants to a point where manipulations can often be targeted to the appropriate tissue and developmental stage. However, during this same time period our ability to analyze the global effects of such modifications on metabolism has lagged behind. We have been very much like the blind men and the elephant; often only able to touch one small portion at a time of the beast we call metabolism!

The good news is that technology continues to hold great promise for the future of plant metabolic engineering. We are now able to analyze the consequences of transgenic or genetic alterations on the expression of thousands or tens of thousands of genes simultaneously. With advances in proteomics we should also be able to simultaneously quantify the levels of many individual proteins or follow post-translational alterations that occur. What are now needed are analogous analytical methods for cataloging the global effects of metabolic engineering on metabolites, enzyme activities and fluxes. Nuclear magnetic resonance and metabolite profiling are likely candidates to fill part of this void (21, 27). Integrating global analyses from transcription to proteins to metabolites may finally allow us to see the elephant in all its glory! A lofty goal to be sure but one has only to look back at the progress in DNA sequencing the past 25 years to realize that anything is possible.

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